Metabolic profiling and in vitro assessment of anthelmintic fractions of *Picria fel-terrae* Lour.

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ABSTRACT

Anthelmintic resistance is widespread in gastrointestinal nematode populations, such that there is a consistent need to search for new anthelmintics. However, the cost of screening for new compounds is high and has a very low success rate. Using the knowledge of traditional healers from Borneo Rainforests (Sarawak, Malaysia), we have previously shown that some traditional medicinal plants are a rich source of potential new anthelmintic drug candidates. In this study, *Picria fel-terrae* Lour. plant extract, which has previously shown promising anthelmintic activities, was fractionated via the use of a solid phase extraction cartridge and each isolated fraction was then tested on free-living nematode *Caenorhabditis elegans* and the parasitic nematode *Haemonchus contortus*. We found that a single fraction was enriched for nematocidal activity, killing $\geq 90\%$ of *C. elegans* adults and inhibiting the motility of exsheathed L3 of *H. contortus*, while having minimal cytotoxic activity in mammalian cell culture. Metabolic profiling and chemometric analysis of the effective fraction indicated medium chained fatty acids and phenolic acids were highly represented.

1. Introduction

Gastrointestinal nematode infections of ruminants remain a major threat to the economic viability of the livestock industry (Nieuwhof and Bishop, 2005; Sackett et al., 2006). The control of these parasitic diseases depends on the use of commercial anthelmintic drugs (Molento et al., 2011), together with management practices (Kahn and Woodgate, 2012; Miller and Waller, 2004; Van wyk et al., 2006, Waller, 1993; Woodgate and Besier, 2010). However, resistance to anthelmintics is widespread in helminth populations (Fleming et al., 2006); therefore, there is a continuous need for the development of novel anthelmintic drugs. The cost of developing new anthelmintic compounds is high, and very few new compounds have been discovered or synthesized in the last decade (Csermely et al., 2013; Mackenzie and Geary, 2013). This is augmented by the fact that nations that suffer most from helminth infections are countries with limited resources to invest in drug discovery research and, as such, are also financially unattractive to the global pharmaceutical industry (Brooker, 2010).

In the last decade, there has been renewed interest in phytomedicine, and many traditional medicinal plants species are being

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tested for pharmacological activities (Agyare et al., 2009; Aremu et al., 2010; Desrivot et al., 2007; Katiki et al., 2011, Ndjonka et al., 2011, Waterman et al., 2010). Although plant-based remedies have been used for centuries in traditional medicine for the treatment of various diseases, including worm infections, there has been limited scientific evaluations of their actual activities, properties and toxicities (Eguale et al., 2011; Kaewintajuk et al., 2010; Ndjonka et al., 2011; Heckendorn et al., 2007; Molan et al., 2003).

Previously, we have shown that traditional medicinal plants are a rich source of anthelmintic drug candidates (Kumarasingha et al., 2014). Plant extracts were selected based on the knowledge of traditional healers from Borneo Rainforests, Sarawak, Malaysia, and screened for anthelmintic compounds in Caenorhabditis elegans (Kumarasingha et al., 2014). The present study reports the fractionation of the most active plant extract, derived from the whole plant of Picria fel-terrae Lour., and identification of a single fraction (designated Fraction 5) that is enriched for anthelmintic activity. P. fel-terrae Lour. is a popular medicinal plant in Asia, and has been studied for its anti-microbial and anti-inflammatory activities (Ahmed and Halaweish, 2014; Huang et al., 1998, 1999; Zou et al., 2006). To the best of our knowledge, apart from our previous work, testing of the anthelmintic activity of this plant has not been reported in the literature.

2. Materials and methods

2.1. Preparation of plant extracts

Plant extracts were prepared at the Sarawak Biodiversity Centre, Kuching, Malaysia. Whole P. fel-terrae Lour. Plants were dried, ground into a powder, extracted into 1:1 v/v dichloromethane:methanol and then concentrated using a rotary evaporator. Before use, the powdered plant extracts were dissolved in absolute ethanol (Merck, Australia) and diluted in M9 buffer (85.6 mM NaCl, 4.2 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 1 mM $MgSO_4$) to achieve the desired concentration (Brenner, 1974). The final ethanol concentration of all dilutions was 1% (v/v).

2.2. Fractionation of P. fel-terrae Lour. whole plant extract

A solid phase extraction (SPE) strata C18-E cartridge (Silica-based sorbent; Phenomenex, USA) was used for sample fractionation. Plant extract (100 mg) was dissolved in 1 ml absolute ethanol and diluted with 19 ml MilliQ water. The column was washed with 20 ml of 100% Acetonitrile (ACN; Sigma, Australia) and then 20 ml of MilliQ water was sent through to condition the column. The dissolved plant extract was loaded on the column and eluted by passing 20 ml of 10%, 25%, 40%, 55%, 70%, 85% and 100% ACN dilution series. The fractions obtained were dried by rotary evaporation. Individual fractions were solubilised in 1 ml of absolute ethanol and 10 μl aliquots were added to 990 μl of M9 to make working solutions.

2.3. C. elegans strains and maintenance

C. elegans "Bristol N2" wild type strain was used for all the experiments. All the strains were grown on Nematode Growth Medium (NGM) in petri dishes containing a lawn of Escherichia coli OP50 (Brenner, 1974). Strains were grown at 20 °C and synchronised populations were obtained by a modified alkaline bleaching method (Lenaerts et al., 2008). Briefly, worm culture plates with eggs and egg-laying adults were washed in M9 and then incubated in a freshly prepared bleaching solution (4 ml commercial bleach, 1 ml 1 M NaOH and 9 ml H_2O) for 3.5 min, followed by washing 3 times with M9. Eggs in M9 were kept on a rotaty shaker at 20 °C

overnight to hatch. First stage larvae (L1s) were put on to NGM plates the next morning and incubated at 20 °C. Young adults were isolated 48 h later. These worms were washed 4 times in M9 before being used in experiments.

2.4. Efficacy of fractions of P. fel-terrae Lour. Whole-plant extracts on C. elegans adults

The 9 fractions collected following SPE fractionation were tested on C. elegans young adults. Assays were performed in 96-well microtitre plates using 150 μl media per well, with 3 replicates per condition (≥10 worms per well) as described previously (Kumarasingha et al., 2014). The plates were covered and kept in a humid chamber on a shaker at 20 °C, and survivors were counted at specific time points for up to 72 h. Worms were considered dead if they were immobile, even after provocation with a platinum wire, and if no pharyngeal pumping was detected. Two conventional anthelmintic drugs, doramectin (Pfizer, Australia) and levamisole (Sigma, Australia) were used as positive controls, and M9 with 1% ethanol was used as a negative control. Each condition was performed in triplicate, and three biological repeats were performed for each experiment.

2.5. Production and storage of H. contortus third-stage larvae (L3s)

Haemonchus contortus (Haecon-5 strain; cf. Schwarz et al., 2013) was maintained in experimental sheep as described previously (Preston et al., 2015) and in accordance with the institutional animal ethics guidelines (permit no. 1111938; The University of Melbourne). To produce L3s, faecal samples were incubated at 27 °C for 7–10 days before larvae were harvested (cf. Schwarz et al., 2013). L3 were stored at 10 °C for up to three months prior to use.

2.6. Exsheathment of L3s

L3s were exsheathed and sterilised by incubation in 0.15% (v/v) sodium hypochlorite (NaClO) at 37 °C for 20 min as described by Preston et al. (2015). Following exsheathment, L3s (designated xL3s) were suspended at a density of 300 xL3 in 50 μl of Luria Bertani medium (LB) supplemented with 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin (Fungizone®,Thermo Fisher Scientific, Australia; designated LB*).

2.7. H. contortus exsheathed third stage larvae (xL3) motility assay

To test for activity, the test fractions (0.01 mg/ml) and the solvent control in LB* with 1% ethanol were arrayed in triplicate in 96 well flat-bottomed plates (Corning, 3650, Life Sciences, USA). Six wells were used for the negative control (LB* + 1% ethanol); 300 L3s were dispensed (in 50 μl) into the wells using a multi-channel pipette and a mini-air pump (Air-pump-S100; Aquatrade, Australia) to keep the parasites suspended, as described by Preston et al. (2015). The perimeter wells were filled with 200 μl of sterile water. Plates were incubated at 38 °C and 10% CO_2 (v/v). Following a 48 h incubation period, the motility of the xL3s was assessed as described previously (Preston et al., 2015). In brief, the plates were agitated on an orbital shaker for 30 min at 38 °C prior to recording a 10 s video of each well using an eyepiece camera (Dino-eye, ANMO Electronic Corporation, Taiwan) attached to a stereo dissecting microscope (Olympus, Japan). Each video was then processed for motility by calculating the changes in pixel intensity using a custom macro in the program ImageJ (1.47v, imagei.nih.gov/ij), as described in detail by Preston et al. (2015).

2.8. Untargeted metabolic profiling and chemometric analysis of the effective fraction

Replicate freeze-dried samples (n = 5) of each fraction (50 ± 2 μg) were prepared for derivatization as previously described (Karpe et al., 2015b; Ng et al., 2012). The stored samples were volatilized by derivatization for application in Gas Chromatography-Mass Spectrometry (GC-MS). Methoxymine HCl (40 μl, 2% w/v in pyridine) was added to the samples, followed by vortexing at 37 °C in a thermomixer (Eppendorf, Australia) at 1400 rpm for 45 min. Silylation was performed by adding 70 μl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 1% Trimethyl-chlorosilane (TMCS) to complete the derivatization. The mixture was then centrifuged at 15,700 × g for 5 min and the supernatant was transferred to GC-MS vials. Pre-derivatized ^{13}C-Sorbitol (Kovats Retention Index = 1918.76, m/z = 620.00 [10 μg/ml, HPLC grade, Sigma-Aldrich, Australia]) was added as the second internal standard at this point in order to verify instrument stability over the run time.

The derivatized samples were analysed using an Agilent 6890B Gas Chromatograph oven coupled to a 5973A Mass spectrometer detector (Agilent Technologies, Australia), as described previously (Beale et al., 2013, 2014). The GC-MS system was fitted with a 30 m DB-5MS column, 0.25 mm ID and 0.25 μm film thickness. All injections were performed at 1:10 split mode with 1.0 μl volume; the oven was held at an initial temperature of 70 °C for 2 min before increasing to 325 °C at 7.5 °C min^{-1}; the final temperature was held for 4.5 min. The transfer line was held at 280 °C and the detector voltages at 1054 V. Total Ion Chromatogram (TIC) mass spectra were acquired at 45–550 m/z, at an acquisition frequency of 1.08 spectra s^{-1}. The ionisation source used was electron ionization (EI) and the energy was 70 eV. The solvent delay time of 7.5 min ensured that the source filament was not saturated and damaged with derivatization reagent. Data acquisition and spectral analysis were performed using MassHunter. Qualitative identification of the compounds was performed according to the Metabolomics Standard Initiative (MSI) Chemical Analysis Workgroup (Sumner et al., 2007) using standard GC-MS reference metabolite libraries of Wiley, NIST 11 and NIST EPA/together with Kovats retention Indices based on the referenced n-alkane retention times (C8-C40 Alkanes Calibration Standard, Sigma-Aldrich, Australia). For peak integration, a 5 point detection, filtering (default settings) was set with a start threshold of 0.2 and a stop threshold of 0.0 for 10 scans per sample.

Chemometric and statistical analysis was undertaken predominately using SIMCA 13.0.3, a chemometric software package (Umetrics AG, Sweden), and post-analysis Fold Change analysis using MetaboAnalyst 2.0 (Xia et al., 2012), an online statistical package (TMIC, Canada). Peak areas were taken into consideration for statistical analyses. Data were normalised with respect to the internal standards (adonitol and ^{13}C-Sorbitol). Chromatography peaks were considered significant where the signal to noise (S/N) ratio was >50, the Fold Change (FC) was >2.0, and p-values were ≤0.05. The calculated p-values were corrected using the Benjamini-Hochberg (BH) adjustment method taking into account a false discovery rate (FDR) threshold of 0.25.

The data generated by mass spectral analyses were normalized with respect to internal standards Relative Standard Deviation (RSD = 5.14%), where a magnitude of 1 fold change (FC) referred to the concentration of 10 mg/l. The normalised data were log transformed and further analysed by Principal Component Analysis (PCA) (Fig. S1) combined with a 'DModX' or 'Distance of observation' plot of active and control fractions (Fig. S2). The two dimensional scatter plot displayed variables with respect to each other and controls. An unsupervised statistical approach using PCA was undertaken on the combined dataset in order to investigate any sub-data grouping, which was not evident (see Fig. S1 and Fig. S2 for the PCA and DModX plots respectively).

To accommodate the outliers and enable differentiation and greater predictability between the groups based on metabolic features, a Partial Least Square-Discriminant Analysis (PLS-DA) was employed (Karpe et al., 2015a, 2015c). PLS-DA is a supervised method used to analyse large datasets and has the ability to assess linear/polynomial correlation between variable matrices by lowering the dimensions of the predictive model, enabling easy discrimination between samples and the metabolite features that cause the discrimination (Wold et al., 2001). The data obtained from PLS-DA were subjected to univariate analyses methods of T-test, fold change analysis and analysis of variance (ANOVA) (Vranova et al., 2013) in MetaboAnalyst 2.0.

2.9. Antimicrobial activity

Orally administered drugs can potentially disrupt the gut microbiota, therefore it was important to test for antimicrobial activity. The most effective fraction was tested against Gram positive (Bacillus cereus ATCC 11778 and Staphylococcus aureus ATCC 12600), Gram negative (Pseudomonas aeruginosa ATCC 10145 and Salmonella entrica serovar Typhimurium ATCC 13311) bacteria for their antimicrobial activity. Overnight cultures were prepared by inoculating approximately 2 ml Nutrient broth (NB) (Oxoid, Australia) with a loopful of microorganism from the slant cultures. All bacterial cultures were incubated at 37 °C overnight under aerobic conditions. For the disk diffusion test, Brain Heart Infusion agar (Oxoid, Australia) was used as the growth medium. The disc diffusion technique involved the following procedure; a sterile cotton-tipped swab was dipped into each bacterial broth and used to inoculate BHI agar plates. The plates were dried for 2–3 min before pre-sterilised discs (6 mm diameter) were placed on the agar surface and 5 μl of the selected fraction were added individually onto the discs. The seeded plates were incubated at 37 °C for 24 h. Tetracycline (30 μg) disks were used as positive controls.

2.10. Cell toxicity of fraction 5

Understanding the cellular toxicity is important in drug discovery and eukaryotic cell cultures are accepted as the model system of choice to get a first approximation of toxicity (Atterwill and Steele, 2009). Mammary epithelial MCF10A cells were seeded in black walled, flat bottom 384 well black walled plates (Corning) at 700 cells/well using a BioTek 406 automated liquid handling dispenser (BioTek, Vermont, USA) in a total volume of 40 μl/well. Cells were cultured in DMEM-F12 containing 5% horse serum (Life Technologies, Australia), 20 ng/ml human epidermal growth factor (EGF, Life Technologies, Australia), 100 ng/ml Cholera toxin (Sigma, Australia), 0.5 μg/ml Hydrocortisone (Sigma, Australia) and 10 μg/ml Insulin (human; Novo Nordisk Pharmaceuticals Pty Ltd). After 24 h incubation at 37 °C, 5% CO_2, the growth medium was aspirated and the cells were treated with Fraction 5 and controls (media ± ethanol (1%) and the chemotherapeutic compound doxorubicin) over an 11 point dose curve and incubated for a further 48 h. On Day 4, cell viability was measured using the Cell-Titer-Glo$^{®}$ luminescent cell viability assay (Promega, Australia). Briefly, 20 μl of CellTiter-Glo$^{®}$ was added to each well and mixed by shaking for 2 min. Plates were incubated for 10 min at room temperature and luminescence was read on a Cytation3 plate reader (BioTek). Doxorubicin, starting at 3 μM concentration was used as the positive control for cell toxicity (data not shown).

2.11. pH stability of fraction 5

pH can influence the stability, wettability, solubility, physical form and particle size of drug compounds. Drug degradation often occurs by hydrolysis that can be dependent on the pH of the medium and therefore pH can significantly alter the stability of a drug formulation (Sigfridsson et al., 2009). The pH of fraction 5 was changed to pH 1 by the addition of HCl (Sigma, Australia). After 1 h, the HCl was neutralized by adding 5 M NaOH (Sigma, Australia) and the pH was adjusted to 7 (Sigma, Australia). The pH-adjusted 40% ACN fraction was then diluted and tested on adult *C. elegans* using the same screening method as described in section 2.4.

3. Results

3.1. Efficacy of fractions from whole plant extracts of P. fel-terrae Lour. on C. elegans adults

We fractionated *P. fel-terrae* Lour. plant extract using a C-18 SPE catridge and all fractions showed some capacity to kill the worms, but four consecutive fractions (eluted with 25%, 40%, 55% and 70% ACN) showed a significant effect on *C. elegans* young adult survival (Fig. 1). Of the four active fractions, the 40% ACN fraction (hereafter mentioned as Fraction 5) demonstrated the greatest efficacy, killing 85—100% of *C. elegans* adults. It is possible that the active compound(s) from Fraction 5 may have been present in the other fractions from slight carry over, however the compound/s with the greatest anthelmintic activity have primarily eluted in Fraction 5.

3.2. Efficacy of fraction 5 on xL3 H. contortus

The anthelmintic activity of Fraction 5 was evaluated on *H. contortus* ex-sheathed L3 motility using the motility index assay. We performed three technical replicates and three biological repeats using the same concentration as with *C. elegans* (0.01 mg/ml). Fraction 5 decreased the motility of *H. contortus* xL3 by almost 50% compared to the negative control (Fig. 2). This suggests the presence of concentrated active compound/s with anthelmintic activity in Fraction 5.

3.3. Metabolic profiling of fraction 5

Untargeted GC-MS analysis of Fraction 5 (active fraction) and Fraction 2 were tested for the identification of any putative anti-nematodal components. This approach was able to identify ca.158 metabolites from both fractions. Fraction 2, the comparatively inactive fraction of the two, was used as the negative control for comparison against Fraction 5. Application of PLS-DA increased the linearity and the overall model predictability (Fig. 3A). The analysis was able to improve the metabolite behavioural distribution, as evident from a distinct metabolite distribution (Fig. 3 B). Overall, of 158 metabolites identified by GC-MS, 132 statistically significant metabolites were common to both fractions (Fig. 4) The metabolites present in fraction 5 were differentiated from fraction 2 by the distribution of their fold change (FC) values (Table 1). The 50 most significant metabolites identified are provided in Table S1.

Medium chained fatty acids (MCFAs) were the highest represented species identified in fraction 5, and included heptadecanoic acid (FC = 283.2), 2, 3-bis [TMS]oxy) butanedioic acid (FC = 66.1) and docosanoic acid (FC = 45.9). Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester or 4-hydroxy benzoic acid was also highly represented in this fraction with a FC value of 51.131, equivalent to a concentration of ~511.3 mg/l. This compound has been identified from various higher plants as a secondary metabolic constituent
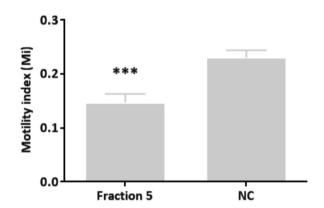
Fig. 2. The effects of fraction 5 (0.01 mg/ml) on exsheathed third-stage larvae (xL3) of *Haemonchus contortus* after 48 h. Results were calculated from three biological and technical replicates (300 worms per replicate). Negative control (NC) represents LB* containing 1% ethanol. Error bar indicates the standard error of the mean (SEM).
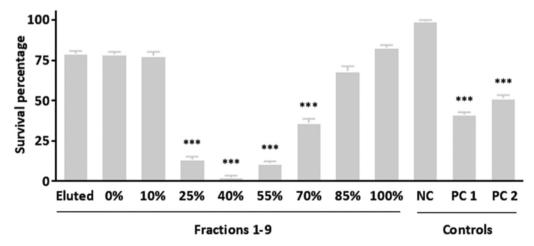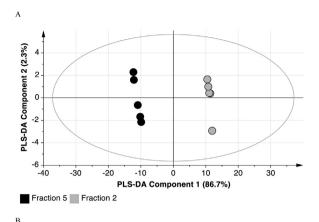
Fig. 1. The effects of fractions (0.01 mg/ml) of *Picria fel-terrae* Lour. plant extracts on young adult *Caenorhabditis elegans* after 72 h. Results were calculated from 3 biological and technical replicates (10 worms per well per replicate). Error bar indicates the standard error of the mean (SEM). Negative control (NC) represents M9 containing 1% ethanol and positive control 1 (PC1) represent doramectin (0.1 mg/ml) containing 1% ethanol and positive control 2 represent levamisole (0.1 mg/ml) containing 1% ethanol. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
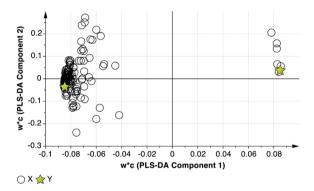
Fig. 3. Geometric profiles of active (Fraction 5) and control (Fraction 2) fractions. **(A)** PLS- DA score scatter plot, where each point on the scatter plot refers to a single sample, with R^2X (cumulative) = 84.3%, R^2Y (cumulative) = 99.9% and Q^2 (cumulative) = 98.2%; and **(B)** PLS- DA loading scatter plot where the yellow five pointed star labels denote the fraction distribution, while the circles represent all the metabolites under consideration and their orientation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Faizi et al., 2011; Lim, 2012). Hydroxycinnamic acid (Trimethylsilyl 3,4-bis(trimethylsiloxy)cinnamate) was also observed at considerable levels in Fraction 5 (FC = 35.136 or 351.4 mg/l). We expect there are other biologically active metabolites in this fraction that were not identified as major constituents in the GC-MS analysis due to detection limits of the system and current mass spectral databases.

3.4. Antimicrobial activity of PE1 fraction 5

Purified compounds previously identified from *P. fel-terrae* plant extracts have shown antimicrobial activity (Huang et al., 1999; Zou et al., 2006). Therefore, we tested Fraction 5 against two Gram positive (*B. cereus* and *S. aureus*) and two Gram negative (*P. aeruginosa* and *S. enterica*) bacteria using the disk diffusion test on Brain Heart Infusion agar. Tetracycline (30 μg) disks were used as positive controls. Interestingly, Fraction 5 did not inhibit growth of any of the tested bacterial strains while as expected tetracycline inhibited the growth of all four bacteria (data not shown), confirming the validity of the test (Galappathie et al., 2014). This indicates that the anthelmintic activity of the compounds in Fraction 5 is highly specific and is not exhibiting general toxicity.

3.5. Mammalian cell toxicity

We used the immortalized human mammary epithelial cell line, MCF10A, to examine the potential toxicity of Fraction 5. Fraction 5 demonstrated minimal and statistically insignificant cytotoxicity at concentrations less than 0.015 mg/ml compared to the negative control (non-treated) (Fig. 5). Levamisole did not show statistically significant cytotoxic activity below 0.5 mg/ml. Since Fraction 5 was used at 0.01 mg/ml for anthelmintic activity testing against both *C. elegans* and *H. contortus*, it could be concluded that there was minimal toxicity at this concentration.

3.6. pH stability of fraction 5

We evaluated the pH stability of Fraction 5 at seven different pH
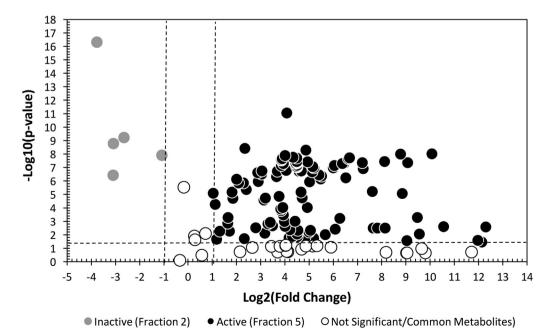

Fig. 4. Volcano plot displaying the differential expressing metabolites in active and control fractions. Important features selected by volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.05. The significant metabolites expressed in Fraction 5 (black circles) and Fraction 2 (grey circles) was taken into consideration for further analyses. Other points on the plot (white circles) were considered as statistically non-significant.

Table 1
Top 20 metabolites characterised by fold change as analysed and identified by GC-MS and univariate and multivariate statistical analyses. Note: The first 14 rows represent metabolites with highest FC values in Fraction 5, while Rows 15–20 represent metabolites with highest FC values in Fraction 2. *Represents the corrected P value using the Benjamini-Hochberg (BH) adjustment taking into account the false discovery rate (FDR) threshold of 0.25. A detailed table is presented in supplementary materials (Table S1).

Metabolites	Kovats retention index	Fold change	P value	Corrected P value*
Fraction 5				
Pentadecanoic acid, n- (1TMS)	1970.853	5078.6	0.0027	0.0049
Glyceric acid, L- (3TMS)	1362.987	4535.5	0.0362	0.0517
1,3-bis(trimethylsilyl)-1,3-diaza-2-germa(II)indane	1980.155	4021.0	0.0268	0.0440
Phosphoric acid (3TMS)	1309.482	3365.1	0.2031	0.2471
Glucopyranoside-6,6-d2, methyl-tetrakis-o-(trimethylsilyl)-	3268.133	1508.4	0.0026	0.0049
Compound 215	2728.253	1083.6	0.0001	0.0003
Compound 220	2728.857	1083.6	0.0001	0.0003
Hexakistrimethylsilyl allitol-3-d1 ether	1908.271	894.9	0.2341	0.2472
Glucose, D- (1MEOX) (5TMS)	1914.547	807.6	0.1098	0.1464
Butane, 1,2,3-tris(trimethylsiloxy)-	1325.525	716.4	0.0005	0.0011
Methyl-α-D-Glucopyranoside (4TMS)	2999.641	542.2	0.0000	0.0003
Fructose, D- (1MEOX) (5TMS)	1889.568	531.5	0.2345	0.2472
Urea (2TMS)	1296.785	527.7	0.0286	0.0440
Gluconic acid-1,5-lactone, D- (4TMS)	1915.996	524.3	0.2348	0.2472
Fraction 2				
Lactic acid, DL- (2TMS)	1070.708	0.7904	0.8200	0.8200
Unknown 1	1725.077	0.4721	$1.26e^{-08}$	$6.3e^{-08}$
25-Hydroxycholecalciferol	1802.400	0.1597	$6.16e^{-10}$	$6.16e^{-09}$
D-Tartaric acid (dTMS)	1774.810	0.1182	$1.72e^{-09}$	$1.15e^{-08}$
Sophorose (dTMS)	1687.649	0.1172	$3.97e^{-07}$	$1.59e^{-06}$
Xylitol (5TMS)	1746.566	0.0735	$5.09e^{-17}$	$1.02e^{-15}$

levels (from 1 to 7) using the *C. elegans* adult viability assays described above. After 72 h, all treated fractions demonstrated statistically similar efficacy compared to the untreated Fraction 5 (Fig. 6), indicating that the active compounds with Fraction 5 are pH stable.

4. Discussion

This study demonstrated that Fraction 5 of *P. fel-terrae* Lour. plant extracts has significant impacts on mortality and motility of *C. elegans* and *H. contortus*, respectively. Moreover, Fraction 5 contains biologically active constituents that were pH stable and showed mimimal cytotoxicity in mammalian cells at the therapeutic concentration used. The GC-MS analysis of Fraction 5 identified a complex mixture of compounds. Although we report the 50 most abundant compounds identified, we recognise the possibility that there may be other potent compounds with good anthelmintic

activities present at lower concentrations but undetectable in our system.

While the biologically active compounds were eluted in Fraction 5, there were other potential bioactive compound/s present in Fractions 4, 6 and 7, although they showed considerably less anthelmintic activity. Of the 158 metabolites identified in Fraction 5, 132 were common to the inactive Fraction 2, leaving 26 specific and highly concentrated molecules that could relate to the effects observed in Fraction 5. It remains unclear what the nature of the anthelmintic activity is in Fraction 5. Further chemical dissection of this fraction will help determine whether a single compound or combination of compounds associate with the anthelmintic activity identified here. Additionally, a limitation of single quadrupole GC-MS based metabolomics is the need to derivatise samples prior to analysis and the difficulty in identifying and characterizing some plant based secondary and tertiary metabolites. As such, any future work analysing compounds that are associated with the
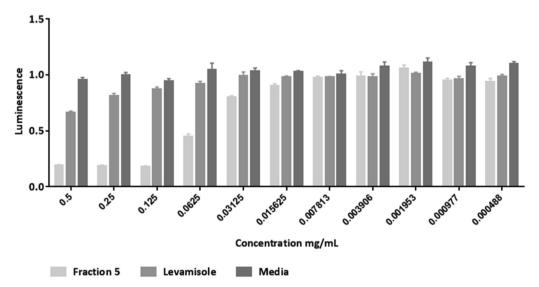

Fig. 5. Analysis of cytotoxicity of Fraction 5. Results were calculated from two biological and 4 technical replicates. Error bar indicates the standard error of the mean (SEM).
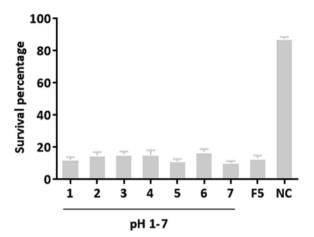
Fig. 6. Effects of pH-treated Fraction 5 (0.01 mg/ml) of *Picria fel-terrae* Lour. plant extracts on young adult *Caenorhabditis elegans* after 72 h. Results were calculated from three biological and technical replicates (10 worms per well per replicate). Error bar indicate the standard error of the mean (SEM). Negative control (NC) represents M9 containing 1% ethanol.

anthelmintic activity of *P. Fel-terrae* Lour. plant extracts would need to consider a suite of complementary techniques (i.e., liquid chromatography (LC), e.g., LC-ToFMS (time of light mass spectrometry), LC × LC-ToFMS and LC-HRT (high resolution time of flight mass spectrometry) and more advanced GC techniques (e.g., GC-ToFMS, GC × GC-ToFMS and GC-HRT). This will likely lead to the discovery and identification of key biomarkers within isolated fractions through the comprehensive metabolomics mapping of *P. Fel-terrae* Lour. plant extracts.

MCFAs were abundant in Fraction 5, and this class of compounds (such as hexanoic, and decanoic acids at 5 mg/L concentrations) have been found to inhibit growth and resultant fermentation (Edwards et al., 1990; Legras et al., 2010). Recently, it has been shown that high concentrations of MCFAs, such as hexadecanoate (palmitic acid) and dodecanoate, caused growth inhibition of *Aspergillus niger* (Karpe et al., 2015b). Furthermore, some MCFAs such as palmitic, oleic, myristic and capric acids have been effective in inhibiting egg hatching by up to 84%, and causing 50% juvenile mortality in root knot nematodes, including *Meloidogyne incognita* (see Zhang et al., 2012) and *M. halpa* (see Sharma et al., 2014).

The metabolite 4-hydroxybenzoic acid is either related to or is a member of the ecatechins family and has been reported to show biological activity (Faizi et al., 2011); 4-hydroxybenzozic acid identified from the floral parts of *Tagetes patula* L has been shown in an in vitro toxicity bioassay to possess anti-nematodal activity (40% mortality at 5 ng/ml) against the free living larvae of the plant cyst nematode *Heterodera zeae* (see Faizi et al., 2011). Likewise, nematicidal and paralysis activities of 4-hydroxybenzoic acid analogues, isolated from chinaberry (*Melia azedarach* L.), have been reported against free living larvae of the plant root-knot nematode *Meloidogyne incognita* ($EC_{50/48h}$ 871 µg/ml) (Aoudia et al., 2012). Hydroxycinnamic acids are secondary metabolites and antioxidants (Olthof et al., 2001), and are an intermediate of lignin biosynthesis in higher plants (Boerjan et al., 2003). Hydroxycinnamic acids have been shown to possess anti-nematodal effects against free living larvae *M. incognita* when isolated from chinaberry (*Melia azedarach*) (EC_{50} > 1000 µg/ml) (see Aoudia et al., 2012) and the plant pathogenic nematode *Pratylenchus scribneri* when isolated from *Lolium arundinaceum* (see Bacetty et al., 2009).

Plant-derived compounds have been shown to have clear efficacy and safety and have been used to develop semi-synthetic or synthetic drugs (Tagboto and Townson, 2001). This study demonstrates that Fraction 5 possesses anthelmintic activity against both free living *C. elegans* and parasitic *H. contortus*. Further analyses should be conducted to understand the mechanism of anthelmintic activity of the active compound/s within Fraction 5.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2016.08.002.

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